"NEW METHOD FOR THE PREPARATION OF 1,5-BIS(4-HYDROXY-3-METOXY-PHENYL)-PENTA-1,4-DIEN-3-ONE AND DERIVATIVES WITH ANTITUMORAL PROPERTIES"

The present patent of Invention refers to a NEW METHOD FOR THE PREPARATION OF 1,5-BIS(4-HYDROXY-3-METHOXYPHENYL)-PENTA-1,4-DIEN-3-ONE AND DERIVATIVES WITH ANTITUMORAL ACTIVITIES.

Bibliographical background about 1,5-bis(4-hydroxy-3-methoxyphenyl)-penta-1,4-dien-3-one compound and derivatives and its method for the preparation.

The compound denominated 1,5-bis(4-hydroxy-3-methoxyphenyl)-penta-1,4-dien-3-one is known since the year 1927 since E. Glaser and E. Tramer for the first time reported its synthesis with a 60% yield (Journal für praktische Chemie, 116, 331-346, 1927) from the vanillin and acetone in the presence of concentrated hydrochloric acid, used as a catalyser.

Further P. Ramanan and M. Rao synthesized this product in 1989 (Indian Journal Pharm. Sci., 51, 207, 1989) from 4-O-methoxymethylvanillin and acetone in an alkaline medium, obtaining a yield of 42% after purifing the same using the thin-layer chromatograph (silica gel).

In 1997 a group integrated by S Sardjiman, et. al. (Eur. Journal Med. Chem. 32, 625-630, 1997) developed a new variant synthesis using equimolecular quantities of vanillin and acetone in the presence of concentrated hydrochloric acid, reporting a raw yield of 89% (without purifing). For this reason the melting point indicated in this procedure was 58 °C less than the one reported by Glaser and Tramer.

M. Artico et. al. also obtained this substance one year later (Journal Med. Chem. 41, 3948-3960, 1998) only obtaining a poor yield of 18%. The melting temperature (114-116 °C) is lower than the one reported by Glaser and Tramer, what it makes think that the compound was not obtained pure in despite of the use of the chromatograph of column.

Additionally we can find in the bibliographic revision the article of the American patent (United States Patent 4,521,629 of June 4, 1985) of N. Cortese et. al. entitled: "Method for the preparation of 1,5-bis-aryl-1,4-pentadien-3-ones". This invention reports to a method of preparing certain bis-arylpentadienones containing fluorine, that

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were used as intermediate compounds for the preparation of insecticidal substituted amidinohydrazones, but it does not protect the products that appear in our patent request.

Furthermore, the following documents of other patents related to this family of organic compounds were found:

1- "Hair tonics containing bis(hydroxyphenyl)pentadienones". Authors of the patent: Morita, Kazuyoshi; Hamada, Kazuto. Company: Kanebo, Ltd, Japan. Country: Jpn. Kokai Tokyo Koho, 7pp. Idiom: Japanese. CA-Number: 134:183278. PI: JP 2001048756, A2 20010220 JP 1999-224982 19990809.

2- "Skin-lightening cosmetics containing distyryl ketones". Author: Morita, Kazuyoshi. Company: Kanebo, Ltd., Japan. Country: Jpn. Kokai Tokyo Koho, 7pp. Idiom: Japanese. CA- Number: 131:149078. PI: JP 11209235 A2 19990803 JP 1998-10414 19980122.

3- "Acidic planting baths and methods for electrodepositing bright and ductile zinc-nickel alloys and additive composition for these baths". Company: McGean-Rohco, Inc., USA. Author: Canaris, Vale-rie M. Country: U.S., 8pp. Idiom: English. CA-Number: 111:183131. PI: US 4832802 A 19890523 US 1988-206017 19880610 EP 346161 A1 19891213 EP 1989-305925 19890612.

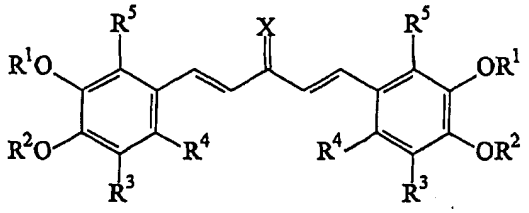
4- "Photopolymerizable compositions". Company: Eastman Kodak Co., USA. Authors: Noonan, John M.; McConkey, Robert C.; Arcesi, J.A.; Rauner; Frederick J. Country: Brit., 19 pp. Idiom: English PI: GB 1425476 A 19760218 GB 1973-3986 19730322 US 3748133 A 19730724 US 1972-237929 19720324.

Neither directly or indirectly none of these four patents are related to the antiproliferate properties shown by 1,5-bis(4-hydroxy-3-methoxyphenyl)-penta-1,4-dien-3-one and its derivatives.

Taking into account the bibliographic revision performed by the company Bypropind Patents and Trademarks based on the tracking in the Chemical Abstracts collection, of Lifescience, of Biomed of Fiocruz library and by us in the Beilstein collection, we have concluded the there is no patent of that compound and its derivatives applicable in the cancer treatment or even the ultrasonic technique in despite of the compound has been synthesized in the year of 1927 and so that the patent request is possible and legitimate.

The present patent of invention reports the antitumoral properties of 1,5-bis(4-hydroxy-3-methoxyphenyl)-penta-1,4-dien-3-one and derivatives and its procedure of preparing.

The sample denominated 37/01 compound was obtained with high yield and purity by the ultrasonic technique presenting cytostatic activity (growth inhibition) in the concentrations evaluated and killer cell activity (cellular death) from the concentration of 0,25 μg/mL against nine different types of human cancer. This compound has a LD_{50}, equals to 8,54 g/Kg. That means this product can be considered itself as practically nontoxic. Doxorubicin, anticarcinogen medicine used as reference in all of these tests, is a product extremely toxic (LD_{50} of 20 mg / Kg) and it does not inhibit the growth of the Mama NCI-ADR cell lines (the one that expresses the phenotype of resistance against multiple drugs), therefore our product presented a strong cytostatic activity.



37 : $R^1 = CH_3$; $R^2 = H$; $R^3 = H$; $R^4 = H$; $R^5 = H$; $X = O$

EHB1 : $R^1 = CH_3$; $R^2 = H_3CCO$; $R^3 = H$; $R^4 = H$; $R^5 = H$; $X = O$

HB6 : $R^1 = CH_3$; $R^2 = CH_2CH=C(CH_3)_2$; $R^3 = H$; $R^4 = H$; $R^5 = H$; $X = O$

HBM1 : $R^1 = CH_3$; $R^2 = CH_3$; $R^3 = H$; $R^4 = H$; $R^5 = H$; $X = O$

HB5 : $R^1 = CH_3$; $R^2 = H$; $R^3 = H$; $R^4 = H$; $R^5 = H$; $X = C(CN)_2$

HB10 : $R^1 = CH_3$; $R^2 = H$; $R^3 = CH_2CH=C(CH_3)_2$; $R^4 = H$; $R^5 = H$; $X = O$

HB11 : $R^1 = CH_3$; $R^2 = H$; $R^3 = H$; $R^4 = H$; $R^5 = H$; $X = C(CN)CO_2C_2H_5$

HB12 : $R^1 = H$; $R^2 = H$; $R^3 = H$; $R^4 = H$; $R^5 = H$; $X = O$

HB13 : $R^1 = CH_3$; $R^2 = H$; $R^3 = Br$; $R^4 = H$; $R^5 = H$; $X = O$

HB14 : $R^1 = CH_3$; $R^2 = CH_2CH=C(CH_3)_2$; $R^3 = Br$; $R^4 = H$; $R^5 = H$; $X = O$

HB15 : $R^1 = H$; $R^2 = H$; $R^3 = CH_2CH=C(CH_3)_2$; $R^4 = CH_2CH=C(CH_3)_2$; $R^5 = H$; $X = O$

Antitumoral activity shown by synthesized compounds.

Legend: NCI460 (Lung tumor); UACC62 (Melanoma); MCF7 (Normal mamma tumor); NCIADR (Mamma tumor which expresses the phenotype resistance against multiple drugs); HT29 (Colon tumor); 786-O (Renal tumor); OVCAR-3 (Ovary tumor); PC-3 (Prostate tumor); K-562 (Leukemia); ED_{50} (effective doses 50 expressed in micrograms by milliliters µg/mL).

Compound	Human Cell Lines tested ED_{50} (µg/mL)								
	UACC-62	MC-7	NCI-ADR	786-O	NCI-460	K-562	PC-03	OVCAR-03	HT29
37	0,03	0,04	0,27	0,65	0,5	0,6	0,41	0,72	0,75
EHB1	1,77	0,45	1,28	0,27	0,7	0,58	0,39	0,57	0,61
HB6	14,16	8,43	3,34	3,64	15,8	2,22	27,99	14	1,87
HBM1	0,75	0,71	0,84	1,19	0,66	0,65	0,82	0,82	0,86
HB5	1,25	1,96	1,11	2,67	2,58	2,84	1,59	43	1,26

Remarks:

All these results can be considered excellent, if we take into reference the ones published in the literature:

Banskota AH, et.al. Chemical Constituents of Brazilian propolis and their cytotoxic activities; J. Nat. Prod. 61, 896-900, 1998

Banskota AH, et.al. Two Novel Cytotoxic Benzofuran Derivatives from Brazilian propolis; J. Nat Prod. 63, 1277-1279, 2000

Kimoto T, et.al. Apoptosis and Suppression of tumor growth by Artepillin C extracted from Brazilian Propolis; Cancer Detect. Prev. 22(6), 505-15, 1998.

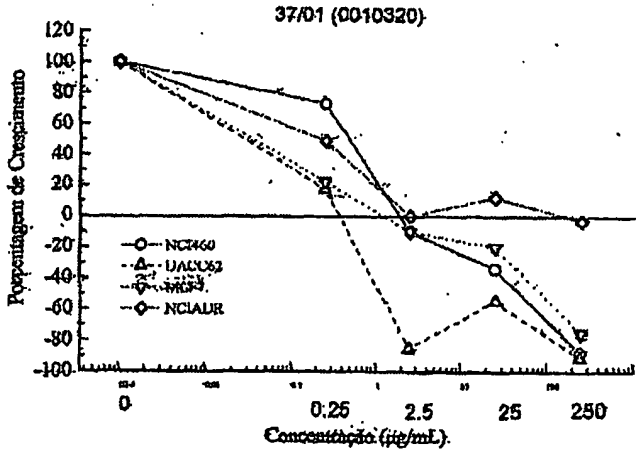
It is observed that the most of our products presented a strong antiproliferate action in concentration ranges (in ppm) much lower than the ones shown by some of the isolated compounds of the Brazilian Propolis.

Cytotoxicity of some isolated compounds of Brazilian Propolis by Banskota and Kimoto.

Name of the compound	HT-1080	Colon L5-26
1 3-hydroxy-2,2-dimethyl-8-prenylchromane-6-propenoic acid	71.53	77.07
2 2,2-dimethyl-8-prenyl-2H-1-benzopyranbenzopyrene-6-yl-2-propenoic acid	46.86	50.22
3 3,5-diprenyl-4-hydroxycinnamic acid	45.47	59.32
4 4-dihydrocinnamoyloxy-3-prenylcinnamic acid	25.94	77.90

We want to point out that the obtained products by our team consist of raw material of our research project, from which we are obtaining new derivatives, guided by the principle of analogy and the results of TOPS-MODE predictions.

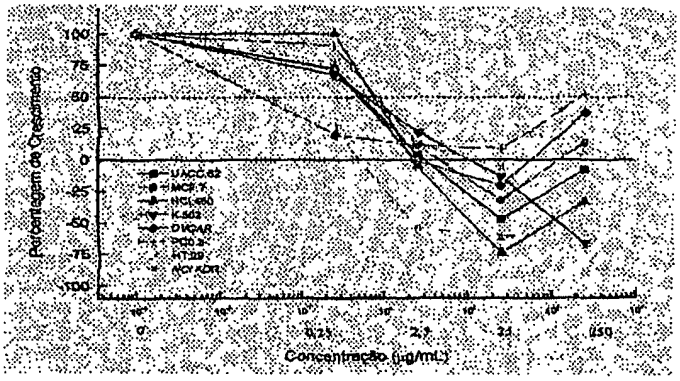
Curves Concentration response of the tested compounds:



37 compound, obtained by means of an organic synthesis procedure, presented cytostatic activity (growth inhibition) for all cell lines and cytotoxic activity (cellular death) for NCI460 (Lung), UACC62 (Melanoma) and MCF7 (Mamma) and NCIADR (Mamma resistant) from 0,25 µg/mL in the first antitumoral testes performed into CPQBA, UNICAMP, September 24, 2001.

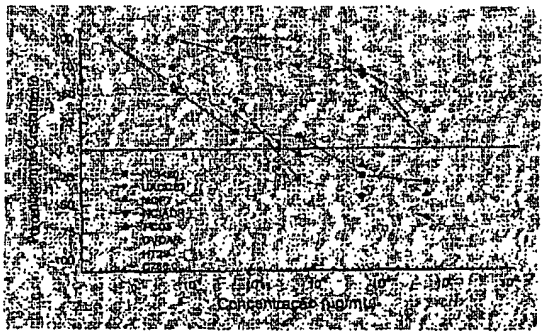
Later these tests of antiproliferate activity were extended to the following cell lines:

Colon; Renal; Ovary; Prostate; Leukemia.



Curve Concentration response of 37.

The results of this compound were compared with Doxorubicin (commercial anticarcinogen used as a standard in these tests) being so similar and in some cases higher than this commercial anticarcinogen.

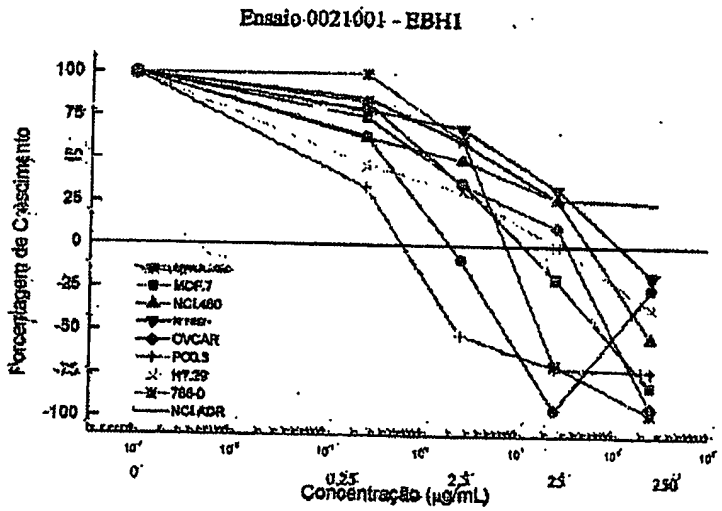


Curve concentration response of Doxorubicin.

For example, our product inhibited the growth of the Mama NCI-ADR cell line (the one that presents the phenotype of resistance against multiple drugs). This result

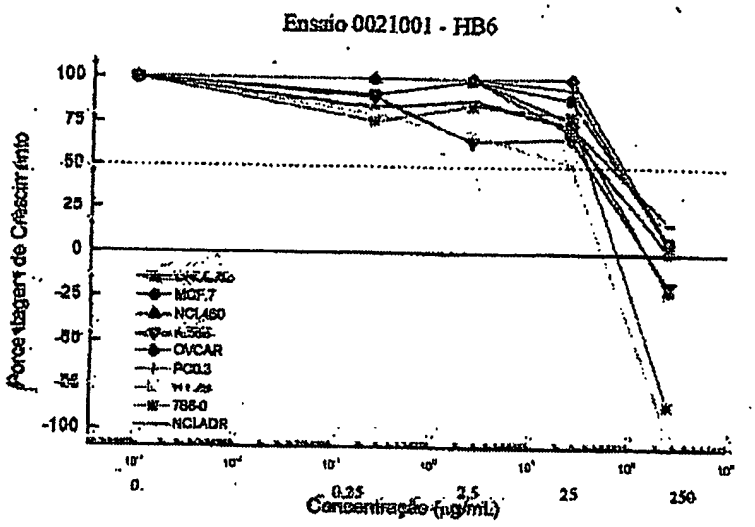
becomes itself very interesting since Doxorubicin, utilized as a positive control, did not inhibit the growth of this human cell line.

The derivatives of 1,5-bis(4-hydroxy-3-methoxyphenyl)-penta-1,4-dien-3-one shown the following antitumoral activity:

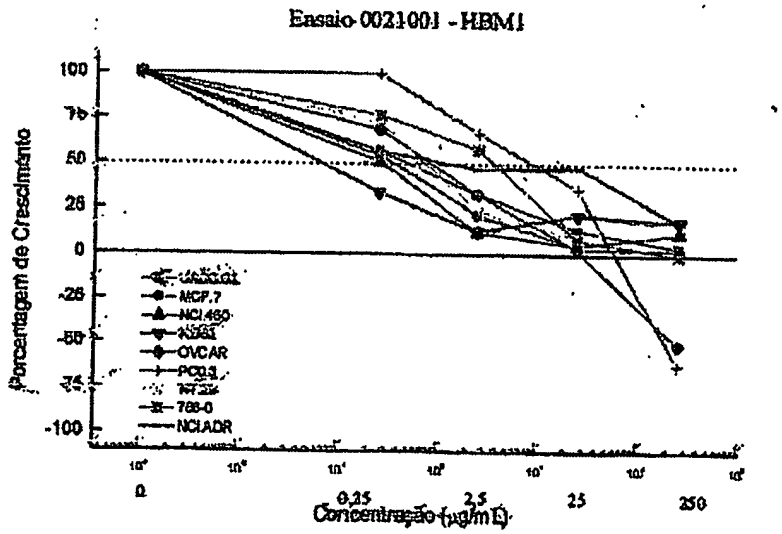


The sample denominated EHB1 compound presented cytostatic activity for all cell lines from the concentration of 0,25 µg/mL and cytotoxic activity from the concentration of 25 µg/mL, excepting NCI-ADR cell line which had only its growth inhibition around 25%. The results showed this sample was not selective for the studied cell lines.

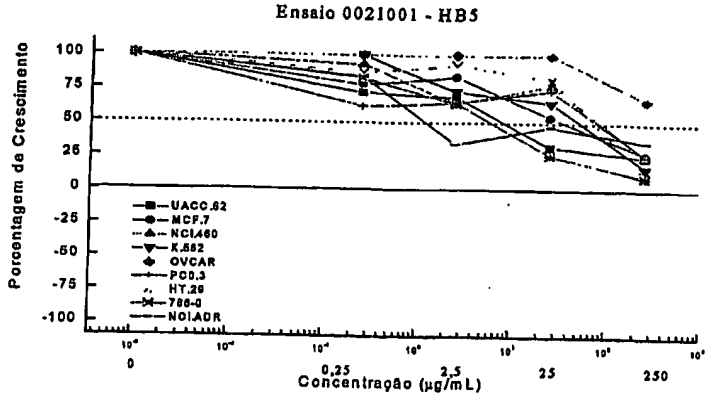
The sample denominated HB6 compound presented cytostatic activity for all cell lines from the concentration of 25 µg/mL and cytotoxic activity in the concentration of 250 µg/mL, for HT-29, 786-0, NCI-ADR and K562 cell lines Furthermore, this sample presented cellular selectivity for HT-29 and 786-0 cell lines.



The sample denominated HBMI compound presented cytostatic activity for all cell lines from the concentration of 0,25 µg/mL and only presented cytotoxic activity for PC-03 and OVCAR-3 cell lines in the concentration of 250 µg/mL.



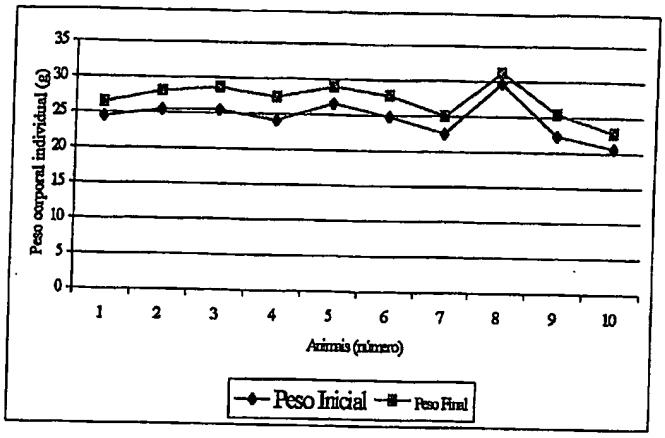
The sample denominated HB5 compound presented moderate cytostatic activity for all cell lines from the concentration of 0,25 µg/mL and did not present cytotoxic activity for any cell lines in the used concentrations.



Also a toxicological test with the sample denominated 37 compound (Acute Toxicity, LD_{50}, via intraperitoneal). The LD_{50} value, evaluated by linear regression, was equal to 8,54 g/Kg, after 14 days of observation. That means this product can be considered itself, according to Loomis, in Principles of Toxicology, as practically nontoxic (compounds with LD_{50} value between 5,0 and 15g / Kg are considered practically nontoxic).

TABLE 1 - Animal's body weight that received 2,5g / Kg of animal body weight of the product denominated "37 COMPOUND", administrated by via intraperitoneal, in the beginning and ending of the acute toxicity test.

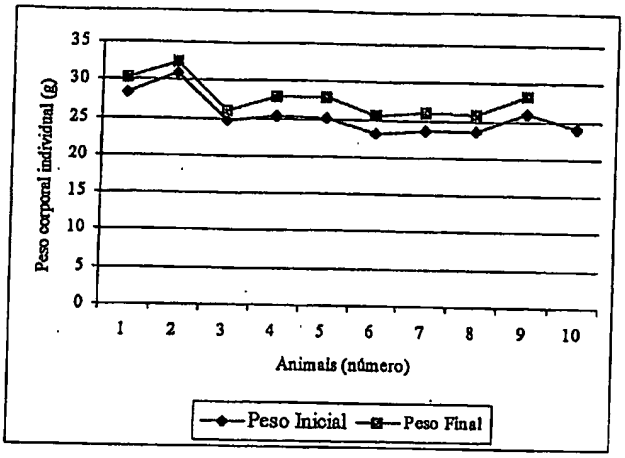
Animal	Initial Weight (g)	Administrated Volume (ml)	Final Weight (g)	Deaths (n)
1	24,5	0,25	26,4	0
2	25,4	0,25	28,0	0
3	25,4	0,25	28,6	0
4	24,1	0,24	27,4	0
5	26,6	0,27	29,0	0
6	24,8	0,25	27,8	0
7	22,5	0,23	25,0	0
8	29,7	0,30	31,2	0
9	22,4	0,22	25,4	0
10	20,6	0,21	22,8	0
Average ± dpm:	24,60 ± 2,51	Average ± dpm:	27,88 ± 1,02	



GRAPHIC 1 - Evolution of the animal's body weight that received 2,5g/Kg of animal body weight of the product denominated "37 COMPOUND", administrated by via intraperitoneal, in the beginning and ending of the acute toxicity test.

TABLE 2 - Animal's body weight that received 3,0g / Kg of animal body weight of the product denominated "37 COMPOUND", administrated by via intraperitoneal, in the beginning and ending of the acute toxicity test.

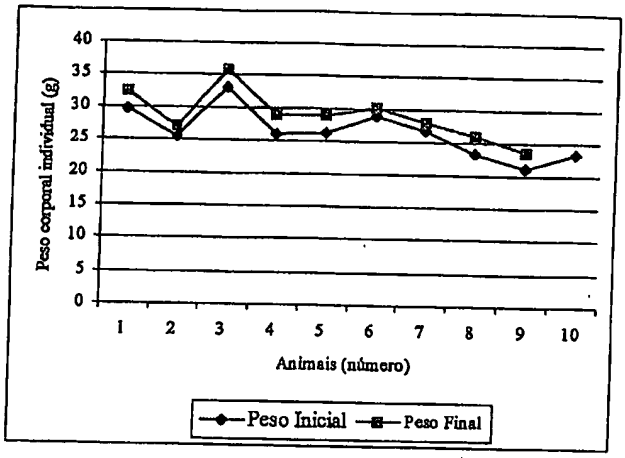
Animal	Initial Weight (g)	Administrated Volume (ml)	Final Weight (g)	Deaths (n)
1	28,3	0,28	30,3	0
2	30,9	0,31	32,5	0
3	24,6	0,25	25,9	0
4	25,4	0,25	28,0	0
5	25,1	0,25	27,9	0
6	23,2	0,23	25,6	0
7	23,6	0,24	26,0	0
8	23,6	0,24	25,8	0
9	26,0	0,26	28,3	0
10	24,0	0,24	Death	1
Average ± dpm:	25,47 ± 2,43	Average ± dpm:	27,81 ± 2,35	



GRAPHIC 2 - Evolution of the animal's body weight that received 3,0g/Kg of animal body weight of the product denominated "37 COMPOUND", administrated by via intraperitoneal, in the beginning and ending of the acute toxicity test.

TABLE 3 - Animal's body weight that received 4,0g / Kg of animal body weight of the product denominated "37 COMPOUND", administrated by via intraperitoneal, in the beginning and ending of the acute toxicity test.

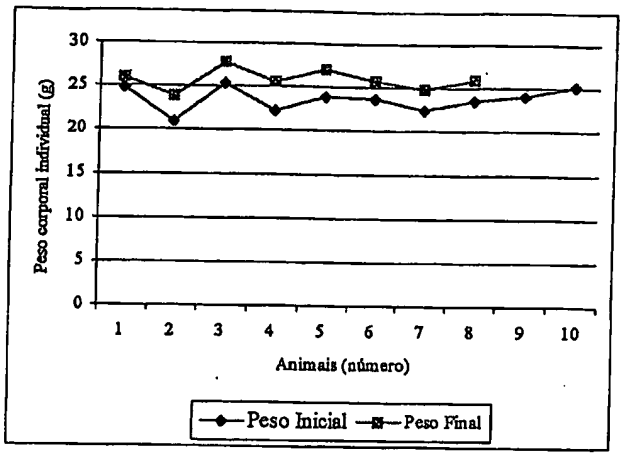
Animal	Initial Weight (g)	Administrated Volume (ml)	Final Weight (g)	Deaths (n)
1	29,8	0,30	32,5	0
2	25,5	0,26	26,9	0
3	33,2	0,33	35,9	0
4	25,9	0,26	28,9	0
5	26,3	0,26	29,0	0
6	28,9	0,29	30,2	0
7	26,7	0,27	28,0	0
8	23,3	0,23	25,9	0
9	21,2	0,21	23,5	0
10	23,3	0,23	Death	1
Average ± dpm:	26,41 ± 3,52	Average ± dpm:	28,98 ± 3,65	



GRAPHIC 3 - Evolution of the animal's body weight that received 4,0g/Kg of animal body weight of the product denominated "37 COMPOUND", administrated by via intraperitoneal, in the beginning and ending of the acute toxicity test.

TABLE 4 - Animal's body weight that received 5,0g / Kg of animal body weight of the product denominated "37 COMPOUND", administrated by via intraperitoneal, in the beginning and ending of the acute toxicity test.

Animal	Initial Weight (g)	Administrated Volume (ml)	Final Weight (g)	Deaths (n)
1	24,9	0,25	25,9	0
2	21,0	0,21	24,0	0
3	25,4	0,25	27,8	0
4	22,2	0,22	25,6	0
5	24,0	0,24	27,0	0
6	23,8	0,24	25,8	0
7	22,5	0,23	24,9	0
8	23,5	0,24	25,9	0
9	24,2	0,24	Death	1
10	25,3	0,25	Death	1
Average ± dpm:	23,68 ± 1,42	Average ± dpm:	25,86 ± 1,17	



GRAPHIC 4 - Evolution of the animal's body weight that received 5,0g/Kg of animal body weight of the product denominated "37 COMPOUND", administrated by via intraperitoneal, in the beginning and ending of the acute toxicity test.

Further this invention is illustrated by means of the following examples of execution:

Example 1.

Preparation of 1,5-bis(4-hydroxy-3-methoxyphenyl)-penta-1,4-dien-3-one.(Method 1)

From vanillin and acetone in a 2:1 molar rate in acid medium, in temperatures that changes between 25 and 60°C, under ultrasonic irradiation conditions in a range from 25 to 40 KHz for a period of 1 to 3 hours further putting the reacted mixture into water / ice until producing the raw product, which dissolves in a solution of sodium hydroxide or potassium hydroxide (between 10-30%) being filtered; the filtrated is treated with hydrochloric acid or sulphuric acid from a concentration between 10-30%, the obtained product being filtered again, finally it is washed with distillated water until obtaining a neutral pH, this operation being repeated until the total purification of the product, not being necessary to perform a new purification of the compound using other procedures such as recrystallization or chromatographic column (the purity was determined by means of the HPLC technique):

Obtained 92% yield of the pure product. Melting point of: 155-156°C.

Method of Preparation 2

A mixture formed of vanillin and acetone in a 2:1 molar rate in acid medium laid during 5-8 days, in temperatures that changes between -10 and 40 °C, further putting the reacted mixture into water / ice until producing the raw product, which dissolves in a solution of sodium hydroxide or potassium hydroxide (between 10-30%) being filtered; the filtrated is treated with hydrochloric acid or sulphuric acid from a concentration between 10-30%, the obtained product being filtered again, finally it is washed with distillated water until obtaining a neutral pH. This operation is repeated until the total purification of the product, not being necessary to perform a new purification of the compound using other procedures such as recrystallization or chromatographic column (the purity was determined by means of the HPLC technique):

Obtained 89% yield of the pure product, melting point of: 155-156°C.

Example 2.

Preparation of 1,5-bis(3-metoxy-4-acetoxyphenyl)-penta-1,4-dien-3-one 1,5-bis(4-hydroxy-3-metoxyphenyl)-penta-1,4-dien-3-one.

It is stirred with an excess of acetic anhydride and sodium acetate. It's heated in a range of temperature between 20 and 110°C for a period of time between 30 minutes to 3 hours. The obtained product is put into distillated water with ice. The precipitate product is recrystallized with hot ethanol. Yield: 58%. Melting point: 150°C.

Example 3.

Preparation of 1,5-Bis[3-metoxy-4-(3-methyl-but-2-eniloxy)phenyl]-penta-1,4-dien-3-one.

A mixture formed of 1,5-bis(4-hydroxy-3-metoxyphenyl)-penta-1,4-dien-3-one (2 mmol) in 10 mL of dimethylformamide and potassium carbonate (6 mmol) is stirred in a range of temperature between 20-50°C during a period of time from 20 to 60 minutes in inert atmosphere (argon or nitrogen). After that 3 mmol of 3-methyl-but-2-enyl bromide with constant stirring. Further the stir is kept for a another period of 5-8 hours with a stream of inert gas pouring all the mixture content into water with ice. It's extracted with chloroform in three occasions with approximately 3 mL of this solvent. The organic phase is washed with a solution of $NaHSO_4$ and then with distillated water. The chloroform phase is dried with sodium sulfate anhydrous, after that the solvent is filtered

and rotoevaporated. The purification of the product is performed utilizing a chromatographic column filled with silica gel gel and using a mixture formed of toluene / ethyl acetate or n-Hexane / ethyl acetate as elution solvents in a appropriate rate. Yield: 53 % of the oil liquid substance.

Example 4.

Preparation of 1,5-Bis(3,4-dimetoxyphenyl)-penta-1,4-dien-3-one.

Method 1

The mixture of 3,4-dimethoxy-benzaldehyd and acetone in equimolecular rate condition in presence of hydrochloric acid was submitted to a ultrasonic bath in the frequence from 25 to 40 KHz between 10-60 minutes in a range of temperature between 25-60°C. Further the obtained product is put into distillated water and ice, filtering the precipitate and washing it with distillated water The aqueous phase is extracted with chloroform and washing the chloroform phase with distillated water and then the chloroform phase is dried with sodium sulfate anhydrous, filtered and rotoevaporated. Yield: 87%.

Method 2

1,5-bis(4-hydroxy-3-metoxyphenyl)-penta-1,4-dien-3-one is stirred with an excess of dimethyl sulfate or methyl iodide in alkaline medium (KOH or NaOH), stirring it in a range of temperature between 25-50°C during a period of time that varies between 5-24 hours. The formed mixture is put into cold water, filtering the formed precipitate, neutralizing it with HCl. Further it's washed with water until neutral pH. The after purification of the product was not necessary. Yield: 85%.

Example 5.

Preparation of 1,5-Bis(3,4-dimetoxyphenyl)-penta-1,4-dien-3-ylidenmalonitrile

In a mixture formed of 1,5-Bis(3,4-dimethoxyphenyl)-penta-1,4-dien-3-one and malonitrile in equimolecular rate condition, amonium acetate, acetic acid and toluene are added following Cope's variant, heating in reflux for a period of time between 5-16 hours or following Knoevenagel's third variant using piperidine as catalyser. The obtained product is put into distillated water and ice, filtering the precipitate and

extracting the aqueous phase with chloroform and washing the chloroform phase with distillated water. Further, the chloroform phase is dried with sodium sulfate anhydrous, filtered and rotoevaporated. Yield: 76%. Melting point: 216°C.

Methodology utilized to perform the antitumoral tests:

REPORT OF THE ANTIPROLIFERATE TEST IN TUMORAL HUMAN CELLS WITH THE SULFORRODAMINA B ESSAY

Cells

The cell lines used in the essays, described in the table 1, were maintained in recipients of 25 cm^2 (Nunc®), with 5 mL of culture medium RPMI 1640 supplemented with 5% bovine foetal serum (RPMI/SFB), at 37°C in atmosphere of 5% of CO_2 and 100% of humidity.

TABLE 1. Cellular lineages used in the assessment essays of the antiproliferate activity

Cellular Type	Code
Lung	NCI460
Mama	MCF-7
	NCI ADR*
Melanoma	UACC-62
Colon	HT 29
Renal	786-0
Ovary	OVCAR-3
Prostate	PC-3
Leukemia	K-562

* cell line that expresses the phenotype of resistance against multiple drugs.

All the procedures described below, were performed under sterile conditions (Laminar Flux Veco®, Class IIB2).

Experimental procedure

100 mL of cells in medium RPMI/SFB with 50 μg/mL of Gentamicin were inoculated in its respectives densities of inoculation, in plates of 96 compartments and incubated for 24 hours at 37°C in atmosphere of 5% of CO_2 and 100% of humidity.

After 24 hours, 100 mL of the test substance were added in the following concentrations of 250; 25; 2,5; 0,2 μg/mL diluted in RPMI/SFB/gentamicin. In this

moment the reading of one plate was performed for the determination of the T0 (cell control in the moment of the adding the samples). The other plates were incubated for 48 hours After this period the experiment was paused by adding the trichlorine acetic acid to further determine the protein content by means of colorimetric essay with the sulforrodamina B.

Dilution of the samples

The stocked solutions were developed by diluting the samples in dimethyl sulfoxide (DMSO) in the concentration of 0,1g/mL. That solution was diluted by 400 times in RPMI/SFB/gentamicina to be added in the plates of 96 compartments being obtained so the a ideal concentration of DMSO (Skehan e cols. 1990).

Essay of the Sulforrodamina B (SRB)

At the end of the test, the plates of 96 compartments were centrifuged for 3 minutes at 2000 rpm, and they were fixed with 50 μL of a solution at 50% of trichloroacetic acid (TCA) at 4 °C. To complete the cellular fixation, the plates were incubated for 1 hour at 4°C.

The plates were submitted to four consecutive washes with distillated water to remore the residues of TCA, medium, SFB and secondary metabolic and maintained at room temperature until the complete drying.

A coloration was performed by the adding of 50 μL SRB at 0,4% (weight/volume) diluted in acetic acid at 1%, and maintained at 4°C for 30 minutes. Further, they were washed by 4 consecutive times with a solution of acetic acid 1%. The residue of the washing of the solution was removed and the plates were dried again at room temperature. The colorant linked to the cellular protein was diluted with a solution of Trizma Base (Sigma®) in concentration of 10 μM and pH 10,5 for 5 minutes in ultrasonic bath. The spectrophotometric of absorbancy reading was performed in 560 nm in a micro plates lector (Labsystems Labsystems Multiskan® MCC/340).

Analysis of the results

The absorbancy averages were assessed having discounted its respective nulls and by means of the formula shown below was determined the growth inhibition (IC) of each tested sample.

T > C the drug did not stimulate the growth, it does not present IG.

If T ≥ T0 but < C, the drug was cytostatic and the formula used is 100 x [(T-To)/(C-T0)]

If T < T0 the drug was cytotoxic and the formula used is 100 x [(T-T0)/(C-T0)]

Considering that T is the absorbency average of the treated cell C is the cell control and T0 is the cell control in the addition day.

The obtained result was subtracted from 100% so obtaining the percentage of growth inhibition. The samples were considered actives since they presented growth inhibition dependant dose higher than 50% and selective lineage, that is, the preferential activity for only one type of tumoral cell or with cytostatic and/or killer cell effect well distinguished among the cell lines.

All the assays were performed in triplicates so that the presented results make reference to a representative experiment. The standard deflection from the average was always lower than 5%.

Methodology utilized to perform the toxicological tests:

Technique

* 10 albinic swiss mouses are used, of the male gender, weighting approximately 25g, for each of the treated groups and control;

* Adaptation period: the animals are maintained in the tests room at least for 07 days before the beginning of the essay;

* The animals are submitted to fast 12 hours before administering the test substance, done by gavage, at the time the animal's body weight is listed.

* After the administration the animals are maintained in observation for a minimum period of 14 days.

* The number of death animals for each one of the doses is listed and the LD_{50} is assessed by Litchfield and Wilcoxon's (1949) method and the animal's body weight is listed at the end of the acute toxicity test.